Liquid Chromatography

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Introduction

The development of selective and sensitive methods for analyses for carbohydrates is one of the most challenging areas of analytical chemistry. The main difficulties in the analysis of carbohydrates arise from their considerable number of isomeric forms due to the various possible configurations of the monosaccharides. Oligo- and polysaccharides are composed of different combinations of them, forming linear or branched polymeric species, many of which may differ only in the position of attachment and anomeric configuration of the glycosidic linkages. Moreover, complex carbohydrates can also be characterized by the presence of various nonglycosyl substituents, which include acetyl, pyruvyl, methyl and sulfate esters and ether, amine and phosphate groups. Finally, methods of analysis for carbohydrates require special attention as they play an important role in many diverse research and industrial domains such as biochemistry, clinical chemistry, biology, pharmacy, biotechnology and food science.

Over the last 25 years, high performance liquid chromatography (HPLC) has been the method of choice for the determination of carbohydrate species, and as a result a large number of HPLC methods have been developed to determine a wide variety of carbohydrate samples.

Alkyl or aminoalkyl bonded silica and polymeric phases, protonated or metal-loaded cation exchange resins in conjunction with refractive index (RI) or low wavelength UV detection, as well as high-performance anion exchange chromatography at high pH (HPAEC), coupled with pulsed amperometric detection (PAD) are the methods commonly used. These different approaches are considered in more detail later.

Polar Phases

Polar silica-based packings, as well as unmodified silica are suitable stationary phases for the separation of sugars and other carbohydrates by hydrophilic interaction chromatography (HILIC), a name proposed in 1990 by Alpert to indicate all chromatographic methods driven by polar interactions, which involve partitioning between the more hydrophobic mobile phase and a layer of mobile phase enriched with water and partially immobilized on the stationary phase. Since 1975 aminopropylsiloxane-bonded silica columns have been used widely for this type of chromatography. The fundamental mechanism governing separation of carbohydrates using aminopropylsiloxane-bonded phases is partition between the mobile phase and the water-enriched solvent associated with the stationary phase.

The most common bonded phases are manufactured by reaction of microparticulate silica (having an average particle diameter of 3 or 5 μm) with organosilanes (for example, 3-aminopropyltrimethoxysilane), to form siloxane bonds. Improvements in column efficiency can be achieved using spherical particles of silica gel, which form more homogeneous beds than those obtained employing irregularly shaped particles.

For separations on aminoalkylsiloxane-bonded silica columns, acetonitrile–water mixtures are usually employed as the mobile phase. The proportion of acetonitrile ranges from 80 to 90% by volume for chromatography of sugars and other carbohydrates of low molecular weight. For example, a mobile phase consisting of 85% acetonitrile in water is useful for the separation of monosaccharides. A low percentage of water in the mobile phase is necessary to increase the interactions between sugars and the amino groups bonded to the stationary phase, improving selectivity and monosaccharide separation. On the other hand, 80% acetonitrile in water allows good separation of di- and trisaccharides differing sufficiently in structure, such as sucrose, maltose, lactose and raffinose. For chromatographic analysis of oligosaccharides of degree of polymerization (dp) above 3, mobile phases containing acetonitrile in lower proportions are required. Under these conditions separations are currently carried out at room temperature and with flow rates from 1 to 2 mL min^{-1}. An example of the effective separation of oligosaccharides obtained by lowering the acetonitrile content in the eluent is shown in **Figure 1**, where about 30 distinct peaks of a partial hydrolysate of (1 → 4)-α-D-glucan from amylose are separated using a mixture of acetonitrile–water (57 : 43, v/v). Although acetonitrile is the most common organic component of the mobile phase, the use of ethyl acetate, together with acetone or methanol have also been proposed.

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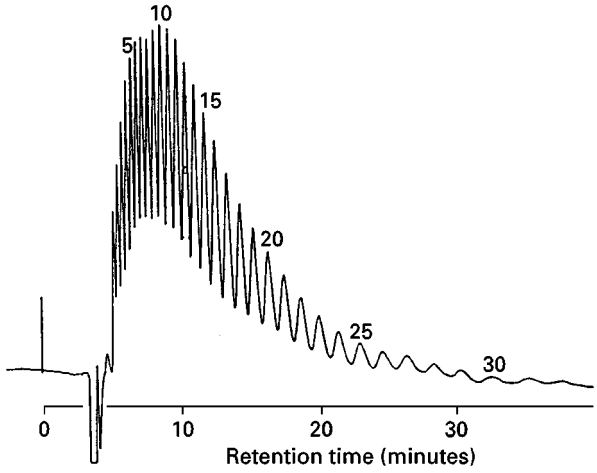

Figure 1 Chromatogram of partial hydrolysate of (1 → 4)-α-D-glucan (amylose). Chromatographic conditions: column, ERC-NH-1171 (200 × 6 mm ID); eluent, acetonitrile–water (57 : 53); flow rate, 1 mL min^{-1}, detector, Shodex RI SE-32 at 1×10^{-5} RI units full scale; temperature ambient. (Reproduced from *Journal of Chromatography* (1985), 321: 151, with the permission of Elsevier Science Publishers.)

Aminopropylsiloxane-bonded silica packings have been widely used in HILIC of sugars and other carbohydrates. However, they have the drawback of limited life, due to the formation of glycosylamines by reaction of the amino groups bonded to the stationary phase with reducing sugars (formation of Shiff's bases). The formation of glycosylamines results in both deactivation of the column and loss of the sugar analytes with an undesirable effect on quantitative analysis. An alternative to the use of these columns is the addition of a small amount (0.01–0.02%, v/v) of a diamino or polyamino compound to the eluent used in HPLC on a silica column, which results in modification of the silica to give it characteristics of an amino propylsiloxane-bonded phase. This approach can be accomplished with various amine modifiers, which must carry at least two amino groups, one being required to bond the modifier to the silica gel via hydrogen bond formation, and one which has to be free to interact with the carbohydrate analytes. Usually *in situ* modified silica columns show a longer life than aminopropylsiloxane-bonded columns, because the amine adsorbed on the silica surface is continuously regenerated by the amine added to the mobile phase. Various polyfunctional amines have been proposed as modifiers, including ethylenediamine, 1,4-diaminobutane, tetraethylenepentamine (TEPA) and piperazine.

Another approach is to replace aminopropylsiloxane-bonded columns with a silica-based packing carrying bonded amide, cyano, diol, polyol or cyclodextrins as alternative stationary phases. These phases have similar selectivity to amino-type phases and do not form Shiff's bases with reducing sugars. Polymer-based columns have also been proposed, increasing application in HILIC of carbohydrates. Although diol-bonded silica, as well as vinylpyridinium polymers have proven to be capable of greater selectivity in carbohydrate separations, aminopropylsiloxane-bonded columns are the most frequently used in HILIC of carbohydrates. A list of some HPLC columns commonly employed in the HILIC of carbohydrates is given in **Table 1**.

Cation Exchangers

HPLC analyses of carbohydrates are most frequently carried out on cation exchange stationary phases consisting of sulfonated polymer-based materials, which can be obtained in various degrees of cross-linking and various particle sizes.

Cation exchangers based on poly(styrene-divinylbenzene) (PS-DVB) copolymers are the most common stationary phases used for carbohydrate separations. Copolymer-based columns offer many advantages for the analysis of carbohydrates and alditols. The PS-DVB copolymers exhibit excellent physical strength, pH stability over a wide range and are not easily subjected to degradation by oxidation, hydrolysis or elevated temperature. Carbohydrate separations on column packed with a cation exchanger PS-DVB resin can be affected by the degree of cross-linking and type of counterion.

PS-DVB resins are relatively rigid gel-type media and separation can take place when the anayltes penetrate at least partially into the matrix. The lower the cross-linking, the more open the structure and more permeable it is to higher molecular weight carbohydrates. For example, a 4% cross-linked resin is suitable to resolve oligosaccharides of high molecular weight, whereas smaller oligosaccharides can be well resolved on an 8% cross-linked resin.

As mentioned earlier, the selectivity of cation exchange columns depends to a large extent on the ionic form of the packing material. Loading of sulfonated resins with various cations produces substantial changes in retention of neutral carbohydrates in several ways, such as the formation of coordination complexes, ion–dipole interactions, ionic hydration and hydrogen bonding. When the resins are loaded with Ca^{2+}, Ag^{+}, Pb^{2+} La^{3+}, marked differences in the retention and selectivity are observed and the mechanism of retention involves the formation of coordination complexes between the carbohydrates and the fixed-metal ions. However, the separation of oligosaccharides is predominantly governed by size-exclusion mechanisms and oligomers elute in order of decreasing molecular weight. The first systematic

Table 1 Examples of silica-based columns and chromatographic conditions employed in carbohydrate separation

Column (particle size, μm) (Supplier)	*Functional group*	*length × ID (mm)*	*Mobile phase*	*Temperature (°C)*	*Flow rate (mL min⁻¹)*	*Detector*	*Applications*
Adsorbosil (5 μm) (Alltech)	Amino	250 × 4.6	Acetonitrile–water (85 : 15)	Ambient	1.0	ELSD	Glucose sucrose lactose melezitose maltotriose
Adsorbsphere (5 μm) (Alltech)	Amino	250 × 4.6	Acetonitrile–water (85 : 15)	Ambient	1.5	RI or ELSD	Fructose glucose sucrose maltose lactose
Alltima NH_2 (5 μm) (Alltech)	Amino	250 × 4.6	Acetonitrile–water (gradient)	Ambient	1.5	ELSD	Maltooligosaccharides
Amino, Spheri-5 (5 μm) (Brownlee)	Amino	250 × 4.6	Acetonitrile–water (75 : 25)	Ambient	1.0	RI	Monodisaccharides
Nucleosil carbohydrate (5 μm) (Macherey-Nagel)	Amino	250 × 4.6	Acetonitrile–water (79 : 21)		1.0	RI	Monooligosaccharides
Hypersil APS-2 (5 μm) (ThermoQuest)	Amino	100 × 3.0	Acetonitrile–water (80 : 20)	Ambient	0.5	RI	Monodisaccharides
Supelcosil LC-NH_2 (5 μm) (Sigma)	Amino	250 × 4.6	Acetonitrile–water (75 : 25)	Ambient	1.0	RI	Ribose arabinose galactoste sucrose maltose isomaltose melezitose raffinose
Ultrasil NH_2 (10 μm) (Beckman)	Amino	250 × 4.6	Acetonitrile–water (75 : 25)	Ambient	1.0	RI	Mono-, di-, trisaccharides
Lichrospher DIOL (10 μm) (Merck)	Diol	250 × 7.0	Acetonitrile–water (gradient)	Ambient	1.0	ELSD	Monosaccharides dextrins
Zorbax NH_2 (5 μm) (Du Pont)	Amino	250 × 4.6	Acetonitrile–water (75 : 25)	Ambient	1.0	RI	Fructose glucose sucrose maltose lactose
Zorbax OH (10 μm) (Du Pont)	Diol	250 × 6.0	Acetonitrile–water (90 : 10)	Ambient	1.0	ELSD	Monosaccharides
Zorbax ODS (10 μm) (Du Pont)	C_{18}	250 × 4.6	Water	60–70°C	1.0	ELSD	dp 2–5
Spheri-5 ODS (5 m) (Brownlee)	C_{18}	100 × 4.6	Water	Ambient	0.5	RI	glucose dp 2–5

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study of the chromatography of sugars and alditols on a cation exchange resin was published in 1975 by Goulding, who showed that the complexing cations Ca^{2+}, Ag^{+}, Sr^{2+} Ba^{2+} and La^{3+} gave longer retention times than the alkali-metal cations. This effect can be attributed to coordination between the metal ions and -OH groups of sugars and alditols and their retention can be correlated with the ability to form relatively strong chelate complexes of the adjacent hydroxyl groups of sugars and alditols with the fixed

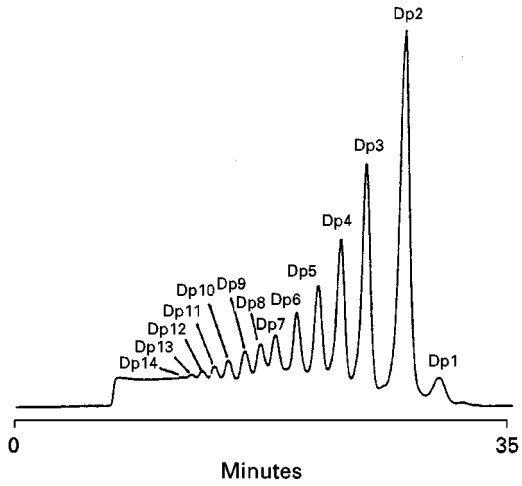

Figure 2 Separation of maltooligosaccharides from dp 14 to dp 2. Chromatographic conditions: column, Rezex RSO-Oligosaccharide (200 × 10 mm ID); mobile phase, water; flow rate, 0.4 mL min^{-1}, temperature, 75°C; detector, RI (with permission of Phenomenex. Torrance, CA, USA).

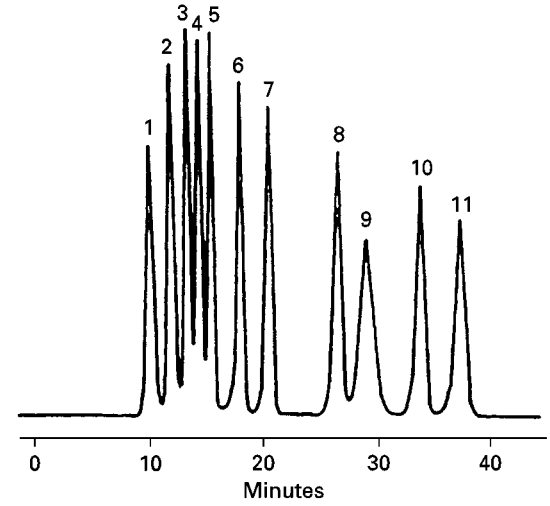

Figure 3 Separation of monosaccharides and alditols. Chromatographic conditions: column, Rezex RPM-Monosaccharide (300 × 7.0 mm ID); mobile phase, water; flow rate, 0.6 mL min^{-1}, temperature, 75°C; detector, RI (with permission of Phenomenex. Torrance, CA, USA).

cation of the resin. For example, stronger binding will occur in sugars where the configuration of the hydroxyl groups at C-1, C-2 and C-3 of the pyranose ring have an axial–equatorial–axial sequence that can form relatively strong tridentate complexes than the binding of sugars, which form complexes with only a pair of axial-equatorial hydroxyl groups. In sulfonated cation-exchange resins carrying Li^+, Na^+, and H^+, there is no evidence that these counterions interact directly with carbohydrates, and their effects can be interpreted simply by ionic hydration. Cation exchange resins carrying calcium ions are the most commonly used for chromatography of sugars allowing good separations of monomeric sugars (pentoses, hexoses), alditols and di-, trisaccharides (**Figure 2**). Cation exchange resins carrying silver ions give better separation of maltooligosaccharides up to a degree of polymerization of 13 glucose units (see **Figure 3**).

Carbohydrate separations on cation exchange resins loaded with various metal ions can be carried out with deionized water as the mobile phase. However, when chromatographic runs are performed at room temperature, peaks are broadened or eluted as a doublet owing to the separation of the two anomeric forms of reducing carbohydrates. As is well known, for each of the ring modifications of a free sugar, two isomers (α and β isomers or anomers) can exist, because a new asymmetric centre is created by ring closure at the reducing carbon atom (see **Figure 4**). The two anomers can be selectively retained on cation exchange resins loaded with various metal ions. For example, when glucose is chromatographed at room temperature on a sulfonated PS-DVB resin column in the hydrogen form, the reducing monosaccharide is eluted as a single peak (**Figure 5**A), whereas with the same column in the calcium form, almost baseline separation of α and β anomers is achieved, indicating that a ligand exchange mechanism is also involved (**Figure 5**B).

The most common approach to avoid the formation of doublets is to increase column temperature. By heating the HPLC column in the calcium form to 85°C, the rate of interconversion between the α and β anomers of glucose is accelerated, and it is eluted as a single peak. Separations are usually performed with a column temperature ranging from 80 to 85°C, though lower temperatures (65°C) may be used. Higher temperatures give faster diffusion and narrower peaks. Conversely, increasing temperature is normally accompanied by a decrease in retention. Organic modifiers such as acetonitrile may be added to the eluent up to a concentration of 30% (v/v) without causing any damage to the PS-DVB matrix. For example, the addition of acetonitrile increases retention of the analytes and may improve resolution of sugar alcohols.

Sulfonated resins in the protonated form are useful for profiling not only monosaccharides and sugar alcohols, but may be useful for analysis of a wide range of organic acids, alone and in combination with carbohydrates, short-chain alcohols, aldehydes and ketones. Selectivity can be alterated by changing the pH as well as the type of mineral acid (H_2SO_4 or HCl at mmol concentration), used as the eluent. Employing these columns, amino sugars may also be separated. Sugars of food interest and sugar alcohols,

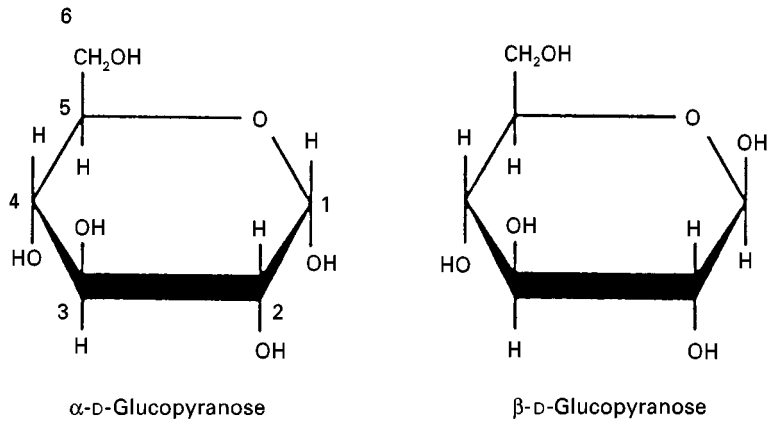

Figure 4 Anomeric configurations of D-glucose.

including sorbitol and mannitol may be separated using pure water as the mobile phase. An example is shown is **Figure 6**, where a mixture of lactose, galactose, mannitol, sorbitol and xylitol are separated on a sulfonated cation exchange resin in protonated form. Furthermore, a separation of lactose, glucose and galactose in a lactose-hydrolysed milk sample is shown in **Figure 7**. Both separations were performed by isocratic elution with water at room temperature. Under these conditions sugar alcohols, as well as reducing sugars are eluted as single sharp peaks.

Tables 2 and **3** list some of the commercially available columns containing sulfonated styrene-divinylbenzene resins in various ionic forms.

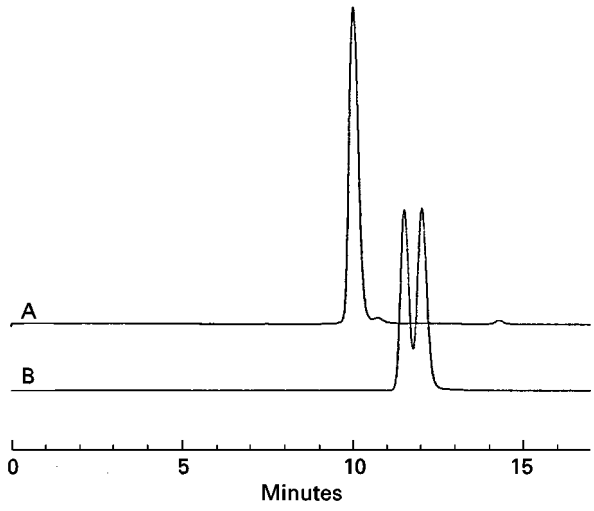

Figure 5 Elution of D-glucose at room temperature. Chromatographic conditions of chromatogram (A) column, PL Hi-Plex H (hydrogen form); dimensions, 300 × 7.7 mm; mobile phase, water; flow rate, 0.6 mL min^{-1}; temperature, 25°C; detection, refractive index detection. Chromatographic conditions of chromatogram (B) column, PL Hi-Plex Ca (calcium form); other conditions as chromatogram A. Both columns from Polymer Laboratories (Shropshire, UK).

Anion Exchangers

Anion exchange chromatography is not a technique commonly associated with analysing neutral carbohydrates. It has been shown that the hydroxyl groups in carbohydrates can form reversible anionic complexes with borate, which can be separated on an anion exchange column. However, this technique is not currently applied to HPLC of mono- and oligosaccharides.

Anion exchange chromatography is basic media allows the separation of carbohydrates without addition of any borate to the mobile phase. The pK_a of neutral carbohydrates usually fall in the range of 12–14, and at high pH their hydroxyl groups are either partially or completed ionized, enabling this class of compounds to be separated as anions by high performance anion exchange chromatography (HPAEC). Moreover, alkaline conditions allow the detection of carbohydrates by PAD at a gold electrode. The compatibility of PAD with gradient elution coupled with the high selectivity of specifically tailored anion exchange stationary phases, allows mixtures of underivatized simple sugars, oligo- and polysaccharides to be separated with good resolution in a single run, and quantified down to picomole levels. Under these conditions, separations cannot be performed using classical silica-based columns due to their poor stability at high pH levels. The columns commercially available are packed with polymer-based anion exchangers produced by proprietary manufacturing processes. These packings include electrostatically latex-coated polymer-based anion exchangers and macroporous polymer-based resins functionalized with quaternary ammonium groups, which have unique selectivity for sugar alcohols.

Monosaccharide separations are usually performed under isocratic conditions and the elution order is inversely correlated with pK_a. Alditols are

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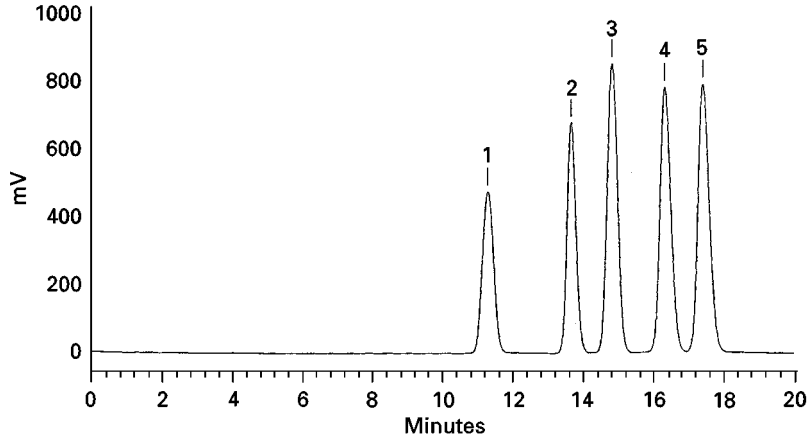

Figure 6 Separation of (1) lactose, (2) galactose, (3) mannitol, (4) sorbitol and (5) xylitol on a sulfonated cation-exchange resin in the hydrogen form. Chromatographic conditions: column, PL Hi-Plex H (8 μm, 300 × 7.7 mm ID Polymer Laboratories Ltd, Shropshire, UK); mobile phase, water; flow rate, 0.4 mL min^{-1} at room temperature; detector, refractive index detection.

less retained than the corresponding reducing sugars as can be readily understood on the basis of acidity. Since the anomeric hydroxyl group is the most acidic among all the OH groups, replacing it with an ordinary OH group (which is less acidic) would naturally result in less retention. For oligosaccharides, retention increases dramatically with increasing molecular mass. HPAEC, followed by PAD detection, has been found to resolve positional isomers of neutral oligosaccharides, which are defined as having the same number, type, sequence, and anomeric configurations as monosaccharides, but differing in the linkage position of a single sugar. Correlation of retention times with the structure of different oligosaccharides may suggest that at least two factors are involved in determining the superior resolution of oligosaccharides by HPAEC: the relative acidity of the hydroxyl groups and the accessibility of oxyanions of the saccharides to the functional groups of the stationary phase.

Addition of a modifier, such as sodium acetate, allows the elution of oligosaccharides in more reasonable times. Acetate anion is used as a strong 'pusher' because it offers rapid equilibration, allowing a

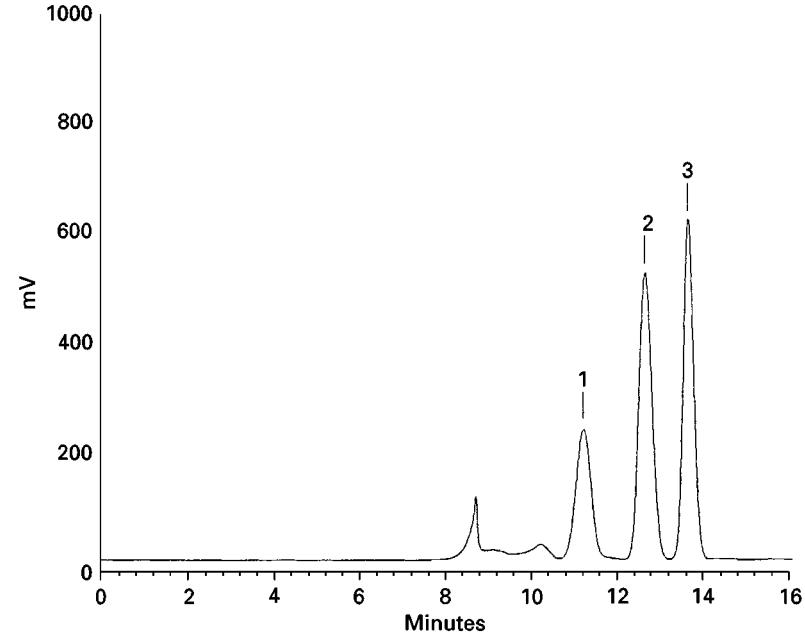

Figure 7 Separation of (1) lactose, (2) glucose and (3) galactose in an UHT lactose-hydrolysed milk. Chromatographic conditions as in Figure 6.

Table 2 Examples of polymeric-based ion-exchanger columns and chromatographic conditions employed in carbohydrate separation

Column (Supplier)	*Matrix (PS-DVB)**	*Length × ID (mm)*	*Mobile phase*	*Temperature (°C)*	*Flow rate (mL min)$^{-1}$*	*Detector*	*Applications*
Cation exchange (Alltech)	Sulfonated (calcium form)	300 × 6.5	Water	90	0.5	RI	Maltotriose, sucrose, glucose, galactose, fructose, mannitol, arabitol, sorbitol
Aminex HPX-87N (Bio-Rad)	(PS-DVB) sulfonated (sodium form)	300 × 7.8	Water	85	0.6	RI	Monosaccharides
Aminex HPX-87K (Bio-Rad)	Sulfonated (potassium form)	300 × 7.8	Water	85	0.6	RI	Monosaccharides
Aminex HPX-87C (Bio-Rad)	Sulfonated (calcium form)	300 × 7.8	Water	85	0.6	RI	Monosaccharides, sugar alcohols
Aminex HPX-87P (Bio-Rad)	Sulfonated (lead form)	300 × 7.8	Water	85	0.6	RI	Cellobiose, glucose, xylose, galactose arabinose mannose
Aminex HPX-42A (Bio-Rad)	Sulfonated (silver form)	300 × 7.8	Water	85	0.4	RI	Oligosaccharides to dp 11
Aminex HPX-87H (Bio-Rad)	Sulfonated (hydrogen form)	300 × 7.8	0.005 N sulfuric acid	50 – 65	0.6	RI	Glucose, ribose, fructose, organic acids
SupelcogelC-611 (Supelco)	Ion-exchange resin containing two different cations	300 × 7.8	10^{-4} N NaOH	60	0.5	RI	Sucrose, glucose, fructose, mannitol, sorbitol, ribose
Nucleogel sugar Pb (Macherey-Nagel)	Sulfonated (calcium form)	300 × 6.5	Water	80	0.4	RI	Sucrose, maltose, glucose, xylose, galactose, arabinose, mannose
Nucleogel ION 300 OA (Macherey-Nagel)	Sulfonated (hydrogen form)	300 × 7.8	Sulfuric acid 0.01 N	30	0.6	RI	Sucrose, glucose, citric acid, fructose, tartaric acid, malic acid, glycerol acetic acid, lactic acid, methanol, ethanol
Nucleogel Sugar Na (Macherey-Nagel)	PS-DVB sulfonated (lead form)	300 × 7.8	Water	85	0.4	RI	Mono oligosaccharides
IOA-1000 (Alltech)	Sulfonated (hydrogen form)	300 × 7.8	0.005 sulfuric acid	65	0.3 – 0.4	RI	Maltotriose, maltose, glucose, fructose, organic acids

*PS-DVB = polystyrene–divinylbenzene copolymer.

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faster elution of strongly retained components without compromizing selectivity and does not interfere with amperometric detection. **Figure 8** is the chromatogram of a sample of starch hydrolysate containing over 20 linear homologous maltooligosaccharides. The separation was obtained by increasing sodium acetate content in the mobile phase by linear gradient elution. On the other hand, under isocratic elution, alditols and mono- and disaccharides can be separated in a single run (**Figure 9**).

As has been shown by several authors in comparative studies, the use of HPAEC-PAD for quantification of neutral monosaccharides, aminosaccharides, glucuronic acids, linear and branched oligosaccharides in complex matrices, is usually superior to HPLC-RI and HPLC-UV in terms of sample

Table 3 Columns with a selectivity comparable to Aminex columns

Aminex column	*Sarasep (length × ID mm) (Interchim)*	*Nucleogel (length × ID mm) (Macherey-Nagel)*	*Polyspher (length × ID mm) (Merck)*	*Sugar-Pak (length × ID mm) (waters)*	*Rezex (length × ID mm) (Phenomenex)*	*Supelcogel (length × ID mm) (Sigma)*
HPX-87N	–	Sugar Na (300 × 7.8)	CH NA (300 × 7.8)	–	RNM (300 × 7.8)	–
HPX-87K	–	–	–	–	RKP (300 × 7.8)	K (300 × 7.8)
HPX-87C	Car-Ca (300 × 7.8)	Sugar Ca (300 × 7.8)	CH CA (300 × 6.5)	SC-1011 SC 1821 (300 × 8.0)	RCM (300 × 7.8)	Ca (300 × 7.8)
HPX-87P	Car-Pb (300 × 7.8)	Sugar Pb (300 × 7.8)	CH PB (300 × 7.8)	SP-0810 (300 × 7.8)	RPM (300 × 7.8)	Pb (300 × 7.8)
HPX-42A	–	–	–	–	RSO (300 × 7.8)	Ag (300 × 7.8)
HPX-87H	Car-H WA-1 (300 × 7.8)	ION 300 OA (300 × 7.8)	OA HY (300 × 6.5)	–	RHM (300 × 7.8)	C-610 H (300 × 7.8)

preparation and sensitivity. In the last five years many significant developments in carbohydrate analysis by HPAEC-PAD have been reported.

Alkylsilica Packings

Octadecylsiloxane bonded silica materials, usually designed for reversed-phase (RP) chromatography are nonpolar stationary phases having relatively strong hydrophobic character. The choice of an RP column with pure water as the eluent is an easy method for HPLC analysis of carbohydrates. However, the mechanism governing retention of carbohydrates is not reversed-phase partition but a form of hydrophobic chromatography, involving van der Waals interactions between carbohydrates and the alkyl chains of

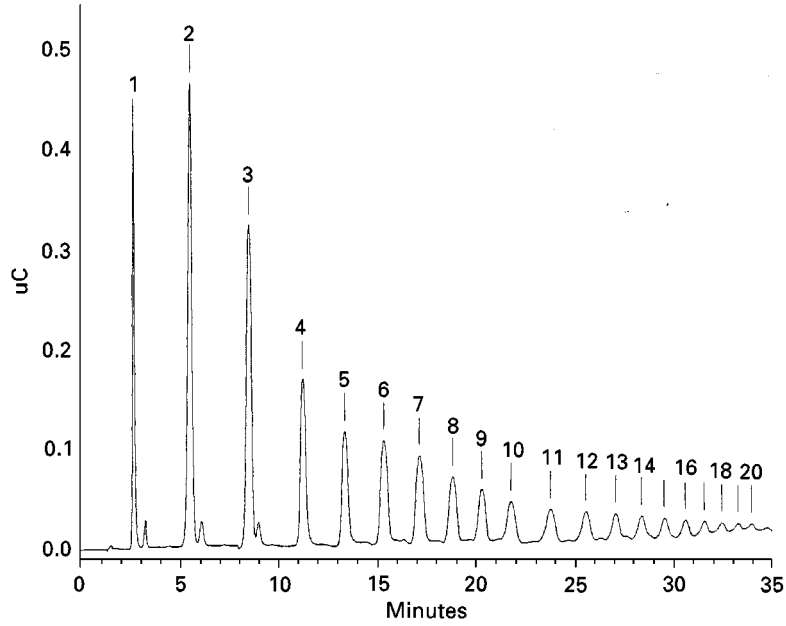

Figure 8 Separation of linear maltooligosaccharide oligomers of up to 20 residues. Chromatographic conditions: column, CarboPac PA 100 (250 × 4.0 mm ID) connected to a CarboPac PA 100 guard column (50 × 4.0 mm ID), all from Dionex, Sunnyvale, CA, USA; eluent A, 50 mM sodium acetate in 120 mM sodium hydroxide; eluent B, 300 mM sodium acetate in 120 mM sodium hydroxide; flow rate, 1.0 mL min^{-1} at room temperature; detector, PED in pulsed amperometric mode. Maltooligosaccharides were eluted by a linear gradient from eluent A to eluent B in 35 minutes. Numbers over peaks indicate dp values.

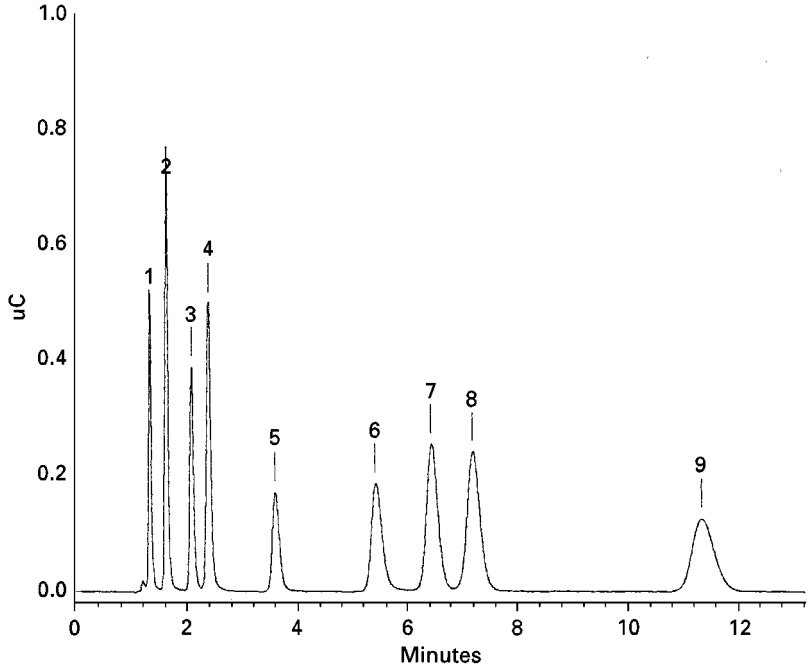

Figure 9 Separation of glycerol (1) xylitol, (2) sorbitol, (3) mannitol, (4) lactitol, (5) maltitol, (6) glucose, (7) fructose, (8) and sucrose, (9) by isocratic elution. Chromatographic conditions: column, CarboPac PA 10 (250 × 4.0 mm ID) connected to a CarboPac PA 10 guard column (50 × 4.0 mm ID), all from Dionex, Sunnyvale, CA, USA; mobile phase, 65 mM sodium hydroxide; flow rate, 1 mL min^{-1} at room temperature; detection, PED in pulsed amperometric mode.

the bonded phase. The first HPLC separation of carbohydrates employing octadecylsiloxane-bonded silica columns was carried out in 1981 using a Dextropak™ (Waters) column specifically developed for the separation of underivatized oligosaccharides. However, separation of monosaccharides was poor. Furthermore, a drawback of the reversed-phase column in carbohydrate separation is the undesired resolution between α and β anomers of the saccharides, which complicates the chromatograms.

Different approaches have been proposed to avoid unwanted double peaks. The most widely used approach is to reduce the oligosaccharide samples with sodium borohydride. In this way for each reducing saccharide the corresponding alditol is eluted as a single peak. As reported earlier, another approach to avoid the formation of doublets is to increase column temperature, which increases the rate of interconversion between the α and β anomers. Cheetham and Teng reported that modification of a Dextropak C_{18} column by adding nonionic Triton X-100 detergent or tetramethylurea to the eluent, avoids the elution of anomeric doublets. The elution of broad peaks or doublets due to resolution of anomers can be precluded by the addition of triethylamine to the eluent. However, under these conditions, some risk of silica degradation is likely.

This drawback may be overcome by the use of reversed-phase packings obtained by introducing octadecyl groups into hydrophilic porous polymers having hydroxyl groups on the surface. Koizumi *et al.* have described the use of an Asahipak™ ODP-50 column packed with C_{18}-bonded vinyl alcohol copolymer gel for the separation of glucooligomers, which were selectively eluted as single peaks using sodium hydroxide at pH 11 as the mobile phase. Under these conditions, the rate of interconversion between the α and β anomers of saccharides was accelerated satisfactorily, avoiding the elution of anomeric doublets. The same polymeric column has been used to separate cyclodextrins and branched cyclodextrins. Columns packed with C_{18}-bonded phases have also found application in ion-pair chromatography with tetrabutylammonium in a buffered medium, for analysis of oligosaccharides containing an acid or sulfate group.

Detection

One shortcoming in the HPLC of carbohydrates is the often inadequate sensitivity and selectivity in the detection process, particularly when they are at a much lower concentration than other compounds present in complex matrices. The analysis of neutral carbohydrates is difficult due to their absence of a chromophore. RI detection is the most popular detection technique for carbohydrates in HPLC but a serious drawback is that it can only be used to monitor

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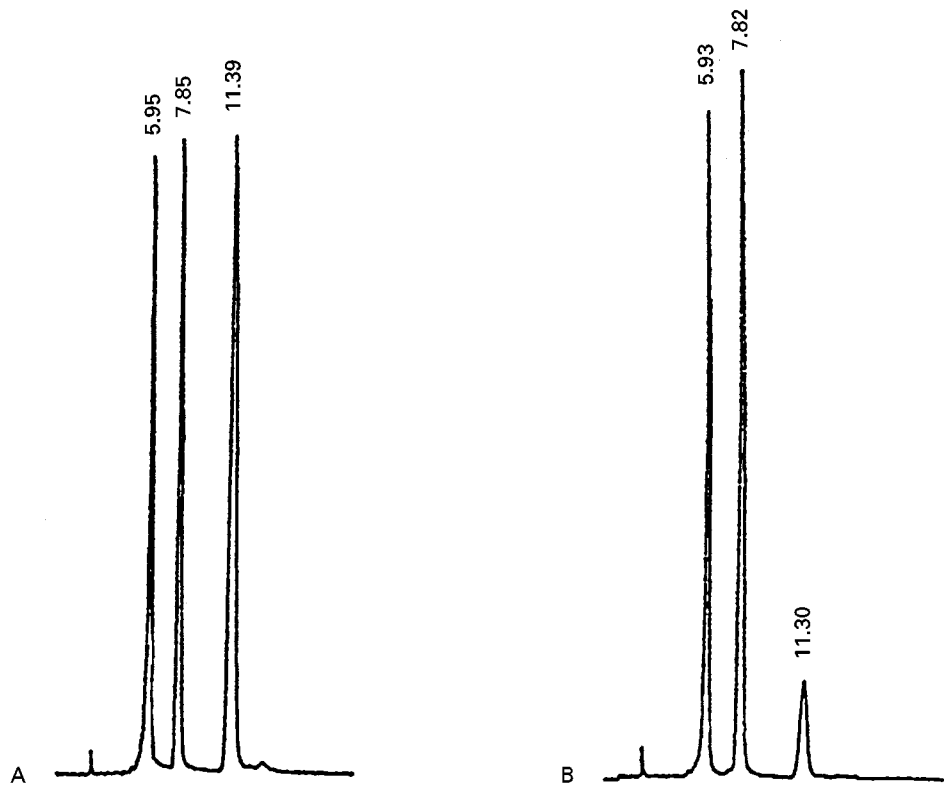

Figure 10 Detection of sugars in fruit juices by ELSD. Chromatographic conditions: column, LiChrosorb Diol (5 μm, 250 × 4.6 mm ID, from Merck); mobile phase, dichloromethane–methanol (82 : 18 v/v); flow rate, 1 mL min^{-1} at room temperature; detector, ELSD; evaporator, 50°C; air inlet pressure, 20 psi. Chromatogram A: fructose ($t_r = 5.95$), glucose ($t_r = 7.85$), sucrose ($t_r = 11.39$) in a blood orange juice. Chromatogram B: fructose ($t_r = 5.93$), glucose ($t_r = 7.82$), sucrose ($t_r = 11.30$) in a grapefruit juice.

isocratic separations. As a substitute for RI, the evaporative light-scattering detector (ELSD) can be used with gradient elution, thus performing better chromatographic separations. In addition, ELSD provides rapid stabilization before operation and improves baseline stability and better sensitivity. The direct detection of analytes is achieved through three consecutive stages, which are independent of each other. The first stage consists in the nebulization of the column effluent with air or nitrogen to produce a finely divided spray, which passes through a heated chamber to vaporize the eluent. During the second stage, solutes less volatile than the eluent create a particle stream that intersects a collimated light beam (mono- or polychromatic, depending on the apparatus design), producing light scattering. The scattered light is then detected by a photomultiplier (or photodiode), where the output is proportional to the amount of solute present over a wide concentration range. Description of the technical performance and configuration of the current commercial ELSD detectors for both HPLC and supercritical fluid chromatography (SFC), have recently been reported by Henry, Dreux *et al.* and Lafosse *et al.* Owing to the nature of light scattering detection, ELSD is especially suitable for solutes which are characterized by a lower volatility than the eluent used for the chromatographic separation. A wide variety of solvents may be used as eluents; highly volatile mobile phases are preferred either as pure solvents or as a combination of two or more. Nevertheless, carbohydrates are more soluble in water than in organic solvents and therefore water is frequently a constituent of the mobile phase.

By coupling the HPLC system with an ELSD detector, carbohydrate separations on bare silica, as well as diol and polyol, bonded phases can be performed by isocratic or gradient elution from mixtures of dichloromethane–methanol. With these organic eluents, separation of mono-, di- and oligosaccharides can be achieved by gradient elution without baseline drift and with a good sensitivity; an example of the analysis of sugars using a diol silica-based column coupled with ELSD detection is reported in **Figure 10**. Fructose, glucose and sucrose are separated from a blood orange juice and a grapefruit juice using a mixture of dichloromethane–methanol (82 : 18 v/v) as the mobile phase. The detection limit is 20 ng for all analysed sugars. Furthermore, the stationary phase employed avoided Schiff's base

formation with reducing sugars and periodic regeneration of the column was not needed. The high chemical stability of the column is demonstrated comparing the reproducibility of the retention times reported in chromatogram A with those reported in chromatogram B, which was performed 100 injections apart. The use of ELSD detection is less favourable when employing metal-bearing cation exchange columns, where carbohydrates are eluted with plain water at high temperature (80–95°C). Since these cationic resin–water systems cause an increase in detector noise at the temperature of the separation. On the other hand, Dreux and Lafosse demonstrated the usefulness of ELSD detection in oligosaccharide and polysaccharide analysis obtained on octadecylsiloxane-bonded silica columns with gradient elution at increasing methanol content in water.

Only few carbohydrates exhibit significant absorbance in the low UV, such as acidic disaccharides released from glycosaminoglycans by enzymatic digestion, which carry an unsaturated uronic acid residue at the nonreducing end that enables their UV detection at an absorbance maximum of 232 nm. Neutral carbohydrates can be analysed by UV detection at wavelengths lower than 200 nm but under these conditions they are subject to interference from other compounds with similar absorption properties. Furthermore, in this UV region acetonitrile also absorbs strongly and therefore the UV detector is unsuitable for use in HILIC of underivatized carbohydrates, where separations are usually performed employing mobile phases rich in this solvent. This drawback can be overcome by either pre- or postcolumn derivatization. The most common chemical derivatization methods allow the conversion of carbohydrates into derivatives that can be detected with an online ultraviolet, visible, or fluorimetric detector. Besides an improvement in the detection properties, the chemical reaction can also enhance the selectivity of the total analytical method. For example, pre-column derivatization of oligosaccharides with a hydrophobic chromophore is the most common approach to enhance the resolution and detection of oligosaccharides into their components by RP-HPLC.

A wide variety of methods have been developed for pre- and postcolumn derivatization of carbohydrates. Comparative advantages and disadvantages of these two approaches have been reviewed by Giese and Honda (see Further Reading).

Although prechromatographic and postcolumn chemical derivatization methods allow the conversion of carbohydrates into either photometrically or fluorimetrically active adducts that can be detected with high sensitivity, the simplicity of sensitive direct detection in HPLC will always be preferred whenever possible.

In HPLC, polar aliphatic compounds containing electroactive groups can be directly detected by pulsed electrochemical detection (PED), a generic term introduced by LaCourse to indicate all detection strategies based on the application of multi-step potential-time waveforms at noble metal electrodes for electrochemical detection.

Amperometric detection is used to measure the current or charge generated by the oxidation or reduction of analytes at the surface of a working electrode in a flow cell. For several classes of molecules, which include carbohydrates and aliphatic alcohols and amines, amperometric detection can only be performed if a repeating sequence of three potentials is applied for specific times to the working electrode. Amperometric detection under the control of a three-step potential-time waveform is known as PAD.

This detection mechanism was first applied in 1981 for the detection of aliphatic alcohols at Pt electrodes and then proposed for the direct determination of carbohydrates when in 1983 Rocklin and Pohl coupled the first commercial detector dedicated to PAD with HPAEC.

In pulsed amperometric detection mode, the working electrode is held at an analytical potential E_1 for a time t_1. At this potential the CHOH groups of carbohydrates are oxidized. The current is measured at the end of the E_1 pulse to allow the charging current to decay, resulting in more accurate measurement of the electroactive species. If only a single potential is applied, the detector response would steadily decrease as the electrode surface became fouled. The electrode surface is oxidatively cleaned by a positive potential E_2 ($E_2 > E_1$) for a time t_2. The voltage on the electrode is then reversed to a strongly reducing potential E_3 for a time t_3 to convert the gold oxide layer at the surface of the electrode back to native gold, thus renewing the surface.

Selection of the potentials for detection, cleaning and conditioning are based on current (i)-potential (E) response from cyclic voltammetry (CV) recorded for triangular waveforms ($E - i$). Extensive studies on the optimization of waveforms for pulsed amperometric detection of carbohydrates have been published recently by LaCourse. Carbohydrates can be detected by PAD at Au electrodes in highly alkaline media. For separations which are not carried out under adequately alkaline conditions, as in the analysis of monosaccharides, the detection requirement of a strong alkaline medium can be satisfied by post-column addition of NaOH (generally in the range 300–500 mM) by means of a pump, a hydraulic device, or membrane reactor.

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Conclusions: Summary of Main Trends

A wide variety of HPLC methods are available to analyse carbohydrates from various complex matrices. Aminopropylsiloxane-bonded silica of small particle diameter (3 μm), amine-bonded vinyl alcohol copolymer packings, diol- and cyclodextrin-bonded silica, as well as macroporous cross-linked vinylpyridinium polymers, are some of the columns of choice for high selectivity in the HILIC of carbohydrates. Furthermore, a large number of columns may be selected to separate carbohydrates on cation exchange resins carrying different counterions. Employing these columns the chromatographic separation is governed by a combination of size exclusion and ligand exchange mechanisms. In oligosaccharide separations, size exclusion is the primary mechanism which involves the binding of hydroxyl groups of the sugars with the fixed counterion of the resin, thus they elute last.

On the other hand, RP-HPLC with plain water as the eluent may be useful for the separation of various underivatized oligosaccharides. The mechanism governing this HPLC method produces separations that complement those given by cation exchange columns and therefore the two techniques should be used in conjunction in isolation and analysis of carbohydrates.

Column instability and low detection sensitivity are the major disadvantages using these HPLC methods. However, column and detection technologies to analyse carbohydrates are in continuous development.

Mono- and oligosaccharides are detected without any derivatization by PAD, which is sensitive and selective for detection of these analytes. The use of HPAEC is now widely accepted as a powerful analytical tool for carbohydrate analysis. However, HPAEC methods require dedicated columns and instrumentation.

See also: **II / Chromatography: Liquid:** Detectors: Evaporative Light Scattering; Detectors: Refractive Index Detectors; Instrumentation; Mechanisms: Reversed Phases. **III / Polysaccharides:** Liquid Chromatography.

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Thin-Layer (Planar) Chromatography

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Introduction

Carbohydrates are some of the more difficult compounds to separate from each other. They primarily differ in the number of carbon atoms, the configuration of the chiral centres and the size of the molecules, that is to say whether they are di-, tri- and oligosaccharides. If two carbohydrates have any one of the three characteristics in common, they can be difficult to separate. There are some carbohydrates that differ by having a special functional group, for example a carboxyl group (giving uronic, onic and aric acids),